Deregulated Syk inhibits differentiation and induces growth factor–independent proliferation of pre–B cells

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The nonreceptor protein spleen tyrosine kinase (Syk) is a key mediator of signal transduction in a variety of cell types, including B lymphocytes. We show that deregulated Syk activity allows growth factor–independent proliferation and transforms bone marrow–derived pre–B cells that are then able to induce leukemia in mice. Syk-transformed pre–B cells show a characteristic pattern of tyrosine phosphorylation, increased c-Myc expression, and defective differentiation. Treatment of Syk-transformed pre–B cells with a novel Syk-specific inhibitor (R406) reduces tyrosine phosphorylation and c-Myc expression. In addition, R406 treatment removes the developmental block and allows the differentiation of the Syk-transformed pre–B cells into immature B cells. Because R406 treatment also prevents the proliferation of c-Myc–transformed pre–B cells, our data indicate that endogenous Syk kinase activity may be required for the survival of pre–B cells transformed by other oncogenes. Collectively, our data suggest that Syk is a protooncogene involved in the transformation of lymphocytes, thus making Syk a potential target for the treatment of leukemia.

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Abbreviations used: Abl, Abelson kinase; ALL, acute lymphocytic leukemia; BCR, B cell receptor; IRES, internal ribosome entry sequence; ITAM, immunoreceptor tyrosine-based activation motif; ITK, inducible T cell kinase; PH, pleckstrin homology; PI3-K, phosphatidylinositol 3–kinase; PLCγ, phospholipase Cγ; RSS, recombination signal sequences; SH2, Src homology 2; SLP-65, SH2 domain–containing leukocyte protein of 65 kD; Syk, spleen tyrosine kinase; TEL, translocated ETS leukemia.

The development of B lymphocytes from hematopoietic progenitor cells in the BM to mature, recirculating B cells can be divided into distinct stages according to the expression of specific marker proteins and the rearrangement status of the Ig H and L chain gene loci (1, 2). Progression through these stages is tightly regulated by signal transduction processes derived from various receptors in the membrane.

One important checkpoint in B cell development is the pre–B cell stage. Productive VDJ recombination at the H chain locus leads to the expression of μ H chain, which is then assembled with the surrogate L chain components λ5 and VpreB and the signal-transducing subunits Ig-α and -β to form the pre–B cell receptor (pre-BCR) (3, 4). Autonomous signaling from the pre-BCR on the cell surface induces cell division, L chain rearrangement, and subsequent differentiation into immature B cells expressing the BCR (5–7). Thus, defects in signal transduction in developing B cells may interfere with normal development and/or enable uncontrolled proliferation, thereby leading to immunodeficiency, autoimmunity, or leukemia.

Signal transduction from the pre-BCR requires recruitment and activation of the spleen tyrosine kinase (Syk) (8, 9). Syk belongs to the Syk/ZAP-70 family of nonreceptor kinases and is characterized by two N-terminal Src homology 2 (SH2) domains and a C-terminal kinase domain, which are separated by a flexible linker (9). Syk is activated by (a) binding via its SH2 domains to phosphorylated immunoreceptor tyrosine-based activation motifs (ITAMs) in the cytoplasmic tails of Ig-α and -β, (b) phosphorylation through Src family kinases, and (c) by autophosphorylation (9). Activated Syk

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phosphorylates several downstream signaling elements, including the adaptor protein SH2 domain–containing leukocyte protein of 65 kD (SLP-65; also known as BLNK or BASH) (8, 9). Phosphorylated SLP-65 provides docking sites for key signaling proteins such as phospholipase Cγ (PLCγ), Vav, Bruton's tyrosine kinase, and growth factor receptor binding protein 2 (10). Binding of these proteins to SLP-65 nucleates a signaling complex that leads to the activation of downstream signaling pathways and the induction of specific transcription factors, thereby altering gene expression and determining cell fate (10).

Mutations in genes encoding signaling proteins and transcription factors are frequently involved in malignant transformation and cancer development. For example, ∼6% of SLP-65$^{-/-}$ mice develop leukemia, and loss of SLP-65 was correlated to development of leukemia in humans (11–13). Although SLP-65 represents a tumor suppressor, several other signaling proteins are actively involved in malignant transformation and are therefore considered protooncogenes. A well-studied example is the Abelson kinase (Abl). Fusion of the *abl* gene to the break point cluster region in the so-called Philadelphia chromosome leads to expression of the deregulated Abl protein (designated BCR-Abl) that is found in chronic myelocytic leukemia, acute myelocytic leukemia, and acute lymphocytic leukemia (ALL) (14). Another example is the transcription factor c-Myc, which regulates the expression of genes involved in the proliferation or differentiation of normal cells and is overexpressed or mutated in a variety of human cancers (15, 16). In Burkitt lymphoma, for instance, the *myc* gene is translocated into the vicinity of the H chain enhancer, resulting in deregulated c-Myc expression that leads to increased proliferation (17).

Several studies suggest an active role of Syk in cancer development. For instance, the TEL-Syk fusion protein was isolated from a patient with myelodysplastic syndrome and has been shown to transform BaF-3 cells in vitro (18). In this case, the dimerization domain of the transcription factor translocated ETS leukemia (TEL; also known as ETV6) is fused to the linker region of Syk by chromosomal translocation t(9;12)(q22;p12), thereby leading to constitutive autophosphorylation and activation of Syk (18, 19). A similar translocation fusing the N-terminal pleckstrin homology (PH) domain and the proline-rich Tec homology domain of the inducible T cell kinase (ITK) to the Syk linker region was recently isolated from patients with peripheral T cell lymphoma (20). In addition, overexpression of Syk was reported in mantle cell lymphoma, and both Syk and ZAP-70 are expressed in ALL (21–23). Furthermore, expression of

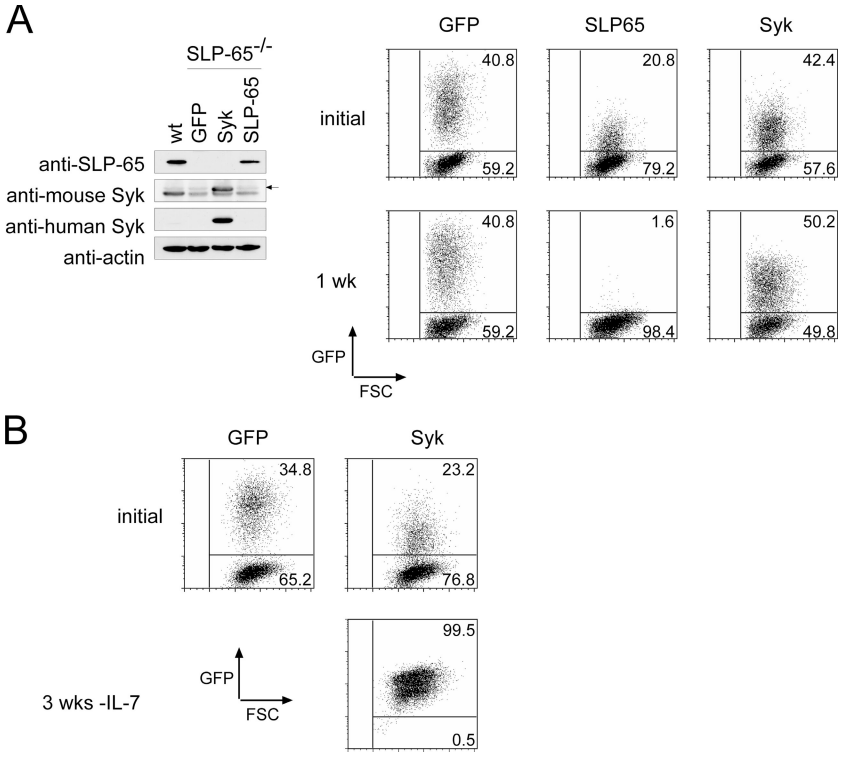

Figure 1. Syk enables growth factor–independent proliferation. (A) SLP65$^{-/-}$ pre–B cells were retrovirally transduced with IRES-GFP vectors encoding either GFP alone, SLP-65, or Syk. (left) Immunoblot analysis for the expression of Syk and SLP-65 in a WT pre–B cell line and transduced SLP-65$^{-/-}$ pre–B cells sorted for GFP expression. (right) Enrichment of Syk-expressing cells. The proportion of GFP^{+} cells in transduced cultures was determined by FACS at 1 d and 1 wk after transduction. Data are representative of three independent experiments. (B) Syk allows proliferation in the absence of IL-7. FACS profiles showing SLP-65$^{-/-}$ pre–B cells transduced with either GFP or Syk and cultured in the absence of IL-7 for 3 wk. Data are representative of five independent experiments. Numbers represent the percentage of cells in the indicated quadrant. FSC, forward scatter.

ITAM sequences has been shown to transform epithelial cells by a mechanism involving Syk, and overexpression of the tumor suppressor PTPROt, a phosphatase regulating Syk activity, was shown to block proliferation of B lymphoma cells (24–26).

On the other hand, several studies indicate that Syk acts as a suppressor of tumor growth and invasiveness in human malignancies. For example, although Syk expression was found in normal breast tissue and noninvasive breast carcinomas, it was not detected in invasive tumors, and its loss has been correlated to a poor prognosis for breast cancer patients (27–29). Although these results were not confirmed by a more extensive study, a correlation between loss of Syk and metastasis formation was found in gastric cancer, melanomas, and a subset of ALL (30–33).

In this paper we address the role of Syk in proliferation and differentiation and show that Syk is a protooncogene able to transform pre–B cells and that Syk activity is also necessary for the proliferation of other leukemia cells, such as those transformed by c-Myc.

RESULTS

Syk transforms pre–B cells in vitro

To investigate the role of Syk in proliferation, we examined its effect on pre–B cell proliferation with that of SLP-65, which acts as a tumor suppressor in pre–B cells (11–13). Therefore, we retrovirally expressed Syk–internal ribosome entry sequence (IRES)–GFP or SLP-65–IRES–GFP in SLP-65$^{-/-}$ pre–B cell lines and monitored the proliferation of GFP^{+} cells compared with nontransduced, GFP^{-} cells of the same

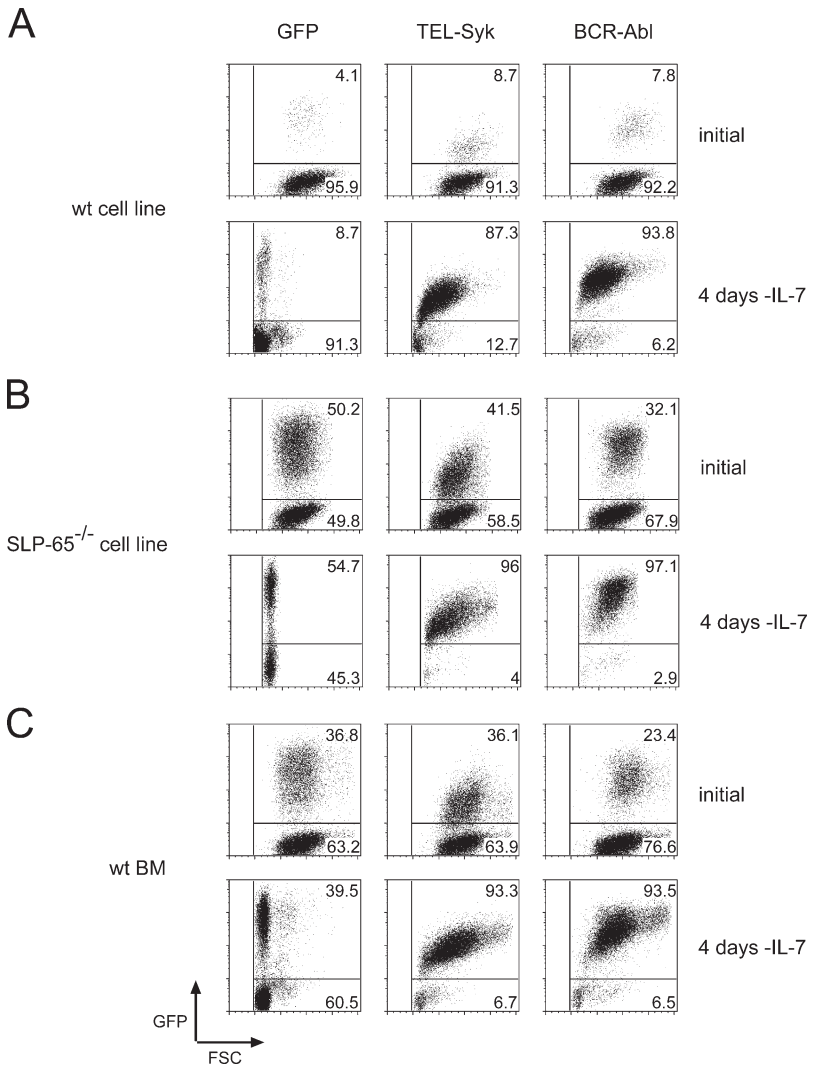

Figure 2. TEL-Syk transforms pre–B cells in vitro. (A) WT pre–B cell lines were retrovirally transduced with IRES-GFP vectors encoding either GFP alone, TEL-Syk, or BCR-Abl. Cells were cultured in the absence of IL-7 and analyzed by FACS at days 1 and 4 after transduction. (B) Transformation of SLP65$^{-/-}$ pre–B cell lines. FACS analysis as described in (A) but using SLP65$^{-/-}$ pre–B cells. (C) Transformation of freshly isolated WT BM-derived pre–B cells. FACS analysis as described in (A) but using freshly isolated BM-derived pre–B cells. Data are representative of five independent experiments. Numbers represent the percentage of cells in the indicated quadrant. FSC, forward scatter.

culture. In line with previous data showing that SLP-65 regulates proliferation, the proportion of SLP-65–expressing cells in such mixed cultures decreased over time (Fig. 1 A). This effect was not caused by SLP-65 overexpression because SLP-65 protein levels were comparable to those in WT cell lines (Fig. 1 A). In contrast, Syk-expressing cells were enriched reproducibly and could be kept in culture for prolonged times, whereas Syk protein levels were elevated as compared with WT and SLP-65$^{-/-}$ cells (Fig. 1 A). This indicates that Syk does not interfere with pre–B cell proliferation and, therefore, does not function as a tumor suppressor. Rather, the accumulation of Syk-expressing cells suggested an involvement of Syk in the activation of pre–B cell proliferation. Because proliferation of mouse pre–B cells depends on the presence of IL-7 as a growth factor, we determined the ability of Syk to induce IL-7–independent proliferation. Withdrawal of IL-7 from cultures transduced with GFP alone blocked proliferation of both the nontransduced and transduced populations and ultimately led to cell death. In contrast, Syk-expressing cells were enriched in cultures without IL-7 and became stable IL-7–independent lines (Fig. 1 B). Similar results were obtained with three out of six tested SLP-65$^{-/-}$ pre–B cell lines and one out of three tested WT pre–B cell lines but not with freshly isolated BM pre–B cells (unpublished data).

As growth factor independence is one hallmark of cellular transformation and is often associated with cancer development, these data indicate that deregulated Syk is involved in the transformation of pre–B cells.

The TEL-Syk fusion protein transforms pre–B cells in vitro

To provide further evidence for the involvement of deregulated Syk in pre–B cell transformation, we tested whether aberrant Syk variants resulting from described chromosomal translocations transform pre–B cells. One such Syk variant is the TEL-Syk fusion protein, which was isolated from a patient with myelodysplastic syndrome and was shown to be constitutively active (18). To test TEL-Syk in pre–B cells, we retrovirally transduced freshly isolated BM pre–B cells and different WT and SLP-65$^{-/-}$ pre–B cell lines with a TEL-Syk–encoding IRES-GFP vector. IRES-GFP vectors encoding GFP alone and the well-studied BCR-Abl oncoprotein were used as negative and positive controls, respectively. Withdrawal of IL-7 from cultures containing transduced and nontransduced cells led to an enrichment of TEL-Syk–expressing cells to ~90% in all tested cell lines and primary BM cultures after 4 d (Fig. 2). Furthermore, nontransduced cells were lost from culture, whereas stable IL-7–independent lines were obtained from all TEL-Syk–expressing cell lines and primary BM cells. Similarly, cells expressing BCR-Abl were enriched to ~90% of the culture after 4 d without IL-7 and gave rise to stable lines, whereas cells expressing GFP alone died out (Fig. 2).

Collectively, these data show that expression of TEL-Syk, like that of BCR-Abl, leads to IL-7–independent proliferation and transformation of pre–B cells in vitro, suggesting that Syk is, analogous to Abl, a protooncogene.

TEL-Syk–transformed cells induce leukemia in mice

The ability to proliferate in the absence of growth factors is a common feature of cancer cells. To test whether TEL-Syk–expressing pre–B cells are able to induce leukemia in mice, we injected a mixture of TEL-Syk–transduced and nontransduced pre–B cells from WT BM into the tail vein of alymphoid RAG-2/γC$^{-/-}$ mice. As controls, we injected a mixture of GFP-transduced and nontransduced cells or BCR-Abl–transformed cells (Fig. 3 A). Mice that received TEL-Syk– or BCR-Abl–expressing cells showed symptoms of disease and developed splenomegaly ~3 wk after injection (Fig. 3 B). FACS analysis of spleens and BM of these animals revealed high numbers of GFP$^+$ CD19$^+$ B cells, whereas GFP$^-$ CD19$^+$ B cells were completely absent (Fig. 3 C). In contrast, mice that received only GFP-transduced cells remained healthy, did not develop splenomegaly, and showed no enrichment of GFP$^+$ cells when compared with the cultures before injection.

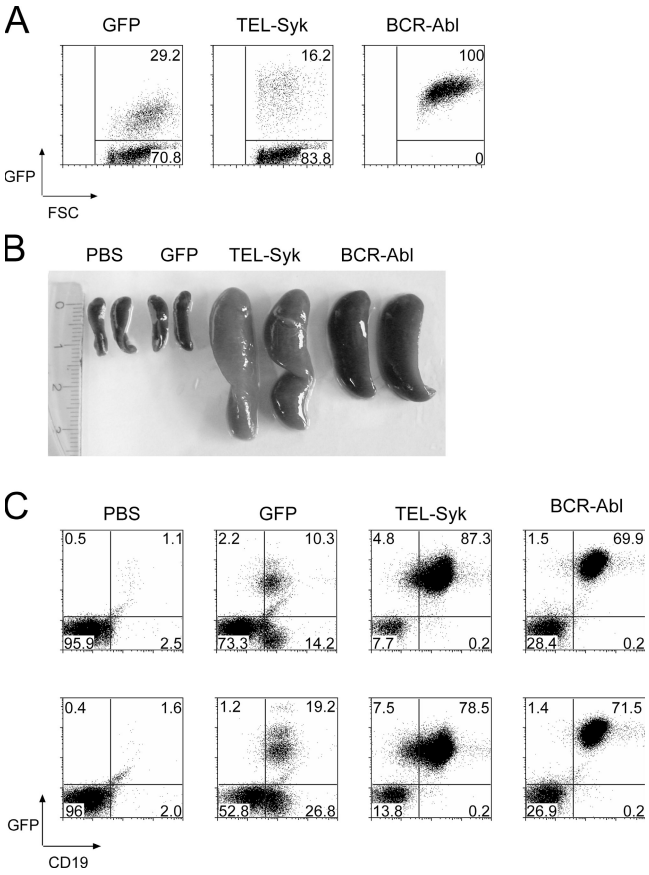

Figure 3. TEL-Syk–expressing cells cause leukemia upon injection in mice. (A) Freshly isolated WT BM-derived pre–B cells (shown in Fig. 2 C) were retrovirally transduced with IRES-GFP vectors for the expression of either GFP alone, TEL-Syk, or BCR-Abl and injected into the tail vein of RAG-2/γC$^{-/-}$ mice. (B) Spleens of the mice 3 wk after injection. (C) FACS profiles showing the expression of GFP and CD19 in cells from the indicated spleens of duplicate mice. Data are representative of five independent experiments. Numbers represent the percentage of cells in the indicated quadrant. FSC, forward scatter.

Collectively, these data show that TEL-Syk–expressing cells proliferate rapidly in vivo, thereby inducing aggressive leukemia, similar to BCR-Abl–transformed pre–B cells.

Syk kinase activity is required for transformation of pre–B cells

To test whether TEL-Syk–induced transformation is dependent on Syk kinase activity, we generated a kinase-negative mutant of TEL-Syk with a lysine to alanine amino acid replacement at position 402 (TEL-SykK402A) in the ATP binding site of the kinase domain (34). Expression of the TEL-SykK402A mutant in different pre–B cell lines and BM pre–B cells did not confer IL-7–independent growth to these cells, suggesting that Syk kinase activity is required for transformation (unpublished data). In line with this, TEL-Syk–transformed SLP-65$^{-/-}$ pre–B cells showed strong tyrosine phosphorylation of specific substrates, including TEL-Syk itself, whereas TEL-SykK402A–expressing cells showed reduced phosphorylation, which was comparable to that of GFP-expressing cells (Fig. 4 A). Although strong tyrosine phosphorylation of distinct substrates was also observed in BCR-Abl–transformed cells, the phosphorylated high molecular mass (>70 kD) proteins differed between BCR-Abl– and TEL-Syk–transformed pre–B cells (Fig. 4 A). To identify the main high molecular mass substrates for TEL-Syk, we immunoprecipitated tyrosine-phosphorylated proteins from lysates of TEL-Syk–transformed pre–B cells, separated them by SDS-PAGE, and silver stained the gels (Fig. 4 B). Analyses of the main bands by mass spectrometry revealed that several bands at 80–83 kD correspond to TEL-Syk itself and that phosphatidylinositol 3–kinase (PI3-K), Cbl, and PLCγ2 are major phosphoproteins in TEL-Syk–transformed pre–B cells.

The specific Syk inhibitor R406 blocks Syk-induced proliferation

Because transformation by TEL-Syk is dependent on Syk kinase activity, we wanted to test whether Syk-specific inhibitors might be used to block the proliferation of Syk-transformed cells. A substance commonly used for inhibition of Syk is piceatannol, while a novel and more specific Syk inhibitor is R406 (35–37). To investigate whether these substances block the proliferation of Syk-transformed cells, we first tested whether they reduce the specific protein phosphorylation observed in Syk-transformed cells. Therefore, we treated TEL-Syk–transformed pre–B cells for various times with R406 and piceatannol and analyzed the pattern of phosphorylation by immunoblotting (Fig. 4 C). As controls, we used the Src family kinase inhibitor PP2 and the Abl inhibitor STI-571 (also known as Gleevec or Imatinib). Remarkably, phosphorylation of the majority of proteins was strongly reduced after 1 min of treatment with R406. Only weakly phosphorylated bands corresponding to TEL-Syk itself remained visible at 60 min, and prolonged incubation with R406 completely abolished phosphorylation (Fig. 4 C and not depicted). Similarly, the increased overall tyrosine phosphorylation in cells transformed by overexpression of Syk was reduced by R406 treatment, whereas BCR-Abl–transformed cells were only weakly affected by R406 (Fig. S1, available at http://www.jem.org/cgi/content/full/jem.20060967/DC1). Treatment with high concentrations of piceatannol (50 μM) also slightly decreased phosphorylation levels, whereas treatment with neither PP2 nor STI-571 affected the overall phosphorylation in TEL-Syk–transformed cells (Fig. 4 C). Treatment with STI-571, however, decreased the phosphorylation levels in BCR-Abl–transformed cells (Fig. S1).

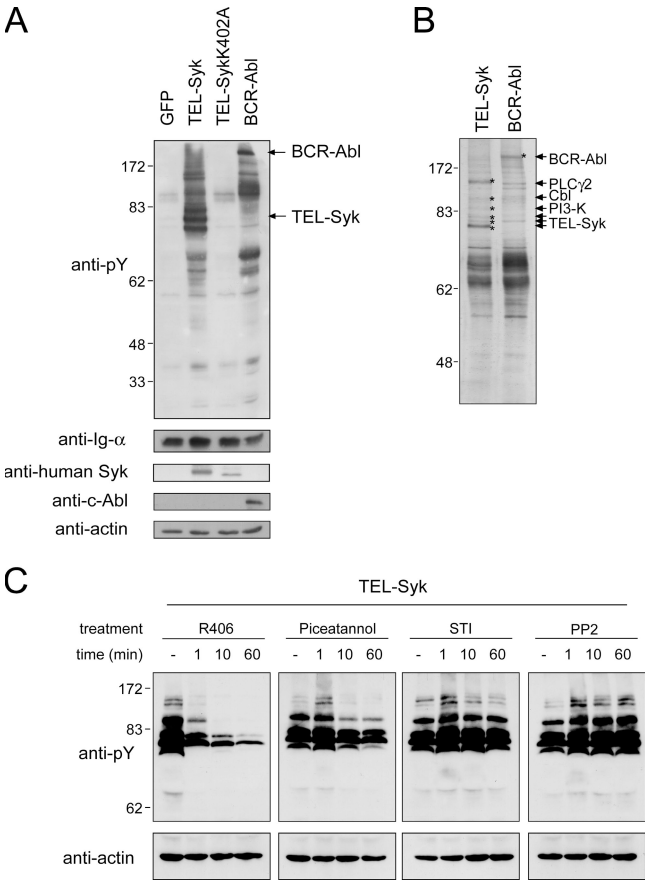

Figure 4. TEL-Syk induces phosphorylation of specific substrates. (A) Immunoblot analysis of pre–B cells transduced with IRES-GFP vectors for the expression of either GFP, TEL-Syk, the kinase-negative TEL-SykK402A, or BCR-Abl. Data are representative of three independent experiments. (B) Silver staining of tyrosine-phosphorylated proteins from TEL-Syk–expressing cells. Total cellular lysates of indicated cells were subjected to immunoprecipitation with antiphosphotyrosine antibodies (4G10). After SDS-PAGE and silver staining, bands marked with an asterisk were cut out and analyzed by mass spectrometry. Proteins identified are indicated next to the respective bands. Data are representative of two independent experiments. (C) The specific Syk inhibitor R406 blocks substrate phosphorylation in TEL-Syk–transformed cells. Shown is an immunoblot analysis of TEL-Syk–transformed cells treated with the Syk inhibitors R406 (2 μM) or piceatannol (50 μM), with the BCR-Abl inhibitor STI-571 (2 μM), or with the Src family kinase inhibitor PP2 (5 μM) for the indicated times. Data are representative of five independent experiments.

We next tested the effect of the Syk inhibitor R406 on the proliferation of TEL-Syk–transformed pre–B cells in vitro. To this end, we examined the DNA content as an indicator for the cell cycle status of TEL-Syk–transformed pre–B cells with and without R406 treatment. In untreated cultures, ~51% of the cells were in S/G2 phase of the cell cycle and were thus proliferating (Fig. 5 A). However, upon treatment with R406, only 4% were proliferating and the majority of the cells (96%) was in G1 phase, whereas neither treatment with STI-571 nor PP2 reduced the proliferation of TEL-Syk–transformed cells (Fig. 5 A). Treatment with R406 also moderately affected proliferation of BCR-Abl–transformed cells, as seen by the reduction from 41 to 29% of cells in S/G2 (Fig. 5 A). On the other hand, treatment with STI-571 reduced the proliferation of BCR-Abl–transformed cells efficiently, as >95% of these cells stop cycling, whereas TEL-Syk–transformed cells were not affected (Fig. 5 A).

In summary, these data show that the proliferation of TEL-Syk–transformed cells is dependent on Syk kinase activity and that inhibiting Syk activity blocks the proliferation of transformed cells.

Inhibition of Syk induces differentiation of TEL-Syk–transformed cells

Proliferation and differentiation of pre–B cells are tightly regulated processes that often interfere with each other. Therefore, we tested whether TEL-Syk–transformed pre–B cells are blocked in differentiation, similar to BCR-Abl–transformed cells, and whether inhibition of Syk activity releases this block and allows differentiation from pre–B cells to

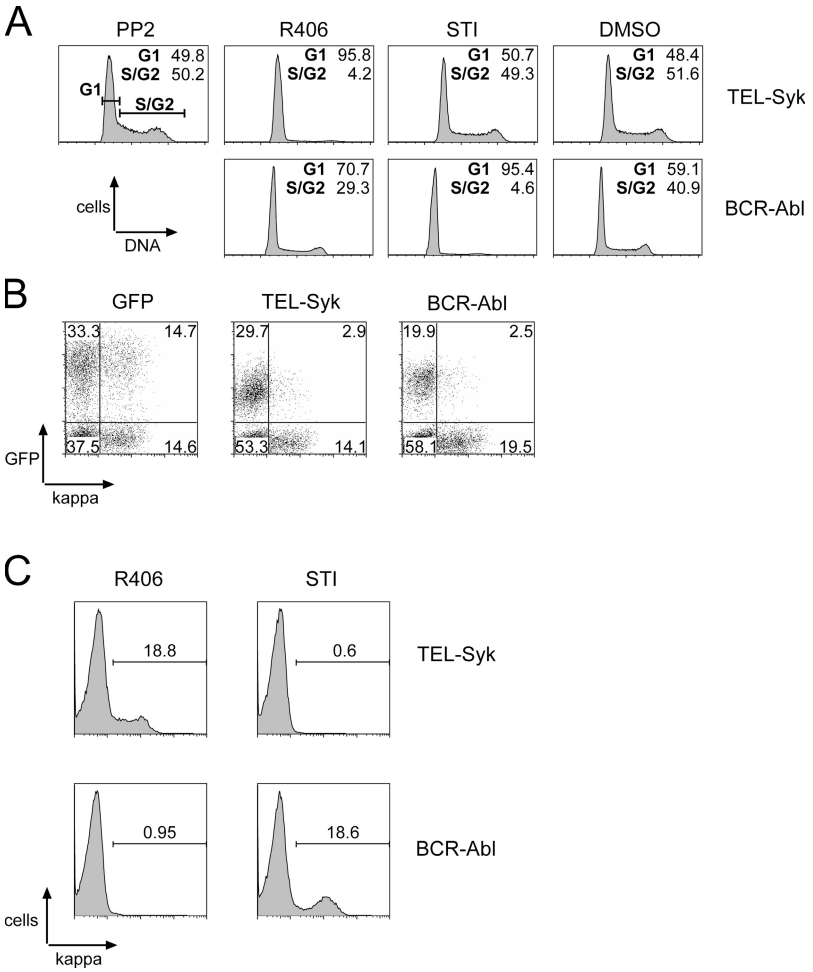

Figure 5. The Syk inhibitor R406 blocks proliferation and induces differentiation of TEL-Syk–transformed cells. (A) Syk inhibition blocks proliferation. Histograms showing the DNA content of TEL-Syk– or BCR-Abl–transformed cells treated for 36 h with DMSO, 2 μM of the Syk inhibitor R406, or 2 μM of the Abl inhibitor STI-571. TEL-Syk– but not BCR-Abl–transformed cells were also treated with 5 μM of the Src family kinase inhibitor PP2. Data are representative of more than five independent experiments. (B) TEL-Syk blocks pre–B cell differentiation. Pre–B cells expressing GFP, TEL-Syk, or BCR-Abl were cultured for 3 d in the absence of IL-7 and analyzed for the expression of κ L chain by FACS. Data are representative of three independent experiments. (C) Inhibition of TEL-Syk with R406 induces differentiation. FACS analysis of κ L chain expression on TEL-Syk– or BCR-Abl–transformed cells treated for 3 d with either 2 μM R406 or 2 μM STI-571. Data are representative of two independent experiments. Numbers represent the percentage of cells in the indicated quadrant or gate.

immature B cells. Indeed, pre–B cells transformed by BCR-Abl or TEL-Syk poorly differentiated into immature, κ L chain–expressing B cells upon withdrawal of IL-7 (Fig. 5 B), whereas treatment with STI-571 or R406 induced differentiation of the respective cells, as demonstrated by κ L chain expression (Fig. 5 C).

However, although cell cycle analyses suggested a moderate effect of R406 on BCR-Abl–transformed cells, R406 treatment failed to induce κ L chain expression in these cells (Fig. 5 C). This discrepancy may be explained by the fact that Ig gene recombination, which is a prerequisite for L chain expression, is often nonproductive and, thus, does not lead to protein expression. To monitor the activation of differentiation more efficiently, we constructed a recombination reporter plasmid with an inverted GFP cassette flanked by recombination signal sequences (RSS) from the κ L chain locus. Induction of differentiation leads to activation of the recombination machinery and subsequent recombination between the two RSS, thereby inverting the GFP cassette and allowing its expression from the nearby promoter (Fig. 6 A). We generated TEL-Syk– and BCR-Abl–transformed pre–B cells containing the GFP recombination reporter plasmid and measured GFP expression after treatment with either R406 or STI-571 for 72 h (Fig. 6 B). All cells from TEL-Syk–transformed cultures treated with R406 showed a reduction in size, and 53% of the cells were GFP^+, demonstrating R406-induced differentiation. After prolonged treatment with R406, all cells died (unpublished data). Treatment with either STI-571 or DMSO did not lead to differentiation of these cells, as seen by unchanged cell size and the lack of GFP expression (Fig. 6 B).

On the other hand, BCR-Abl–transformed cells differentiated efficiently upon treatment with STI-571, as demonstrated by 42% GFP^+ cells. In addition, the GFP recombination reporter confirmed the minor effect of R406 on BCR-Abl–transformed cells, as 4% of these cells turned GFP^+ upon R406 treatment (Fig. 6 B).

In summary, these data show that counteracting Syk-induced proliferation by inhibiting Syk kinase activity induces differentiation of pre–B cells into immature, κ L chain–expressing B cells.

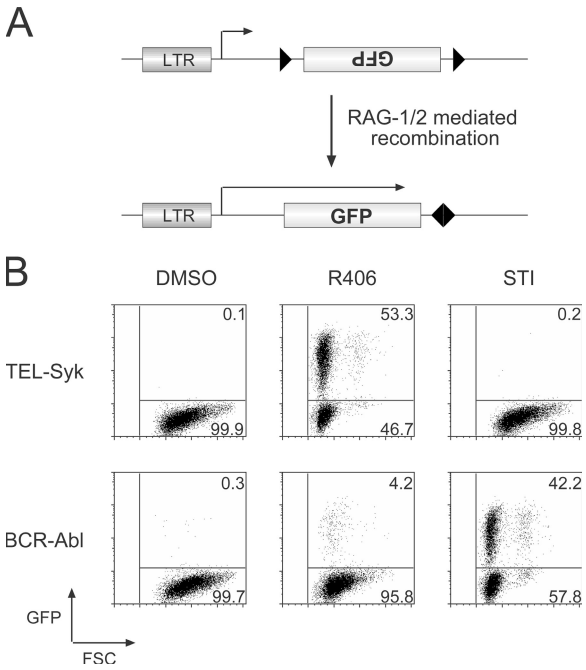

Figure 6. A GFP-based system to measure Syk kinase inhibition. (A) Structure of the recombination reporter plasmid. Induction of RAG-1/2 expression by differentiation-inducing stimuli leads to recombination of the two RSS (black triangles) flanking the inverted GFP cDNA. Flipping the GFP cassette brings the start codon close to the LTR, thereby enabling GFP expression. (B) FACS analysis showing GFP expression in pre-B cells transformed by TEL-Syk or BCR-Abl and containing the recombination reporter plasmid. Cells were treated for 3 d with 2 μM R406 or 2 μM STI-571. Data are representative of three independent experiments. Numbers represent the percentage of cells in the indicated quadrant. FSC, forward scatter; LTR, long terminal repeat.

The proliferation of c-Myc–transformed cells depends on Syk

The experiments presented in the previous sections suggest that Syk is a protooncogene that can promote pre–B cell transformation and cancer development. As cancer cells are often characterized by elevated expression levels of the transcription factor c-Myc (15, 16), we analyzed c-Myc expression in Syk-transformed pre–B cells. Western blot analysis showed that, similar to BCR-Abl–transformed cells (38, 39), c-Myc expression was elevated in Syk- or TEL-Syk–transformed cells compared with cells expressing only GFP (Fig. 7 A). Treatment of TEL-Syk–transformed cells with R406, but not with STI-571, decreased the expression of c-Myc, indicating that Syk is involved in c-Myc regulation (Fig. 7 A).

To investigate whether Syk-induced up-regulation of c-Myc is sufficient for transformation, we retrovirally expressed c-Myc–IRES–GFP in pre–B cells. Upon withdrawal of IL-7 from the culture medium, only c-Myc–expressing GFP^+ pre–B cells survived (Fig. S2 A, available at http://www.jem.org/cgi/content/full/jem.20060967/DC1). Furthermore, injection of the c-Myc–transformed pre–B cells into RAG-2/$\gamma C^{-/-}$ mice induced leukemia, demonstrating the oncogenic capacity of c-Myc (Fig. S2 A). We then treated the c-Myc–transformed cells with R406 and STI-571 and analyzed proliferation by monitoring the DNA content. c-Myc–transformed cells were extremely sensitive to Syk inhibition, as observed by a reduction of proliferating cells from 33% in untreated cultures to 6% in cultures treated with R406 (Fig. 7 B). Furthermore, PP2-mediated inhibition of Src family kinases, which were shown to participate in the pre-BCR–dependent activation of Syk (40–42), reduced the proliferation of cells transformed by c-Myc (Fig. S2 B). On the other hand, treatment with STI-571 did not affect proliferation (Fig. 7 B). These data suggest that c-Myc–transformed cells require constitutive proliferation signals mediated by Syk. Because the pre-BCR is the main focus for Syk activation

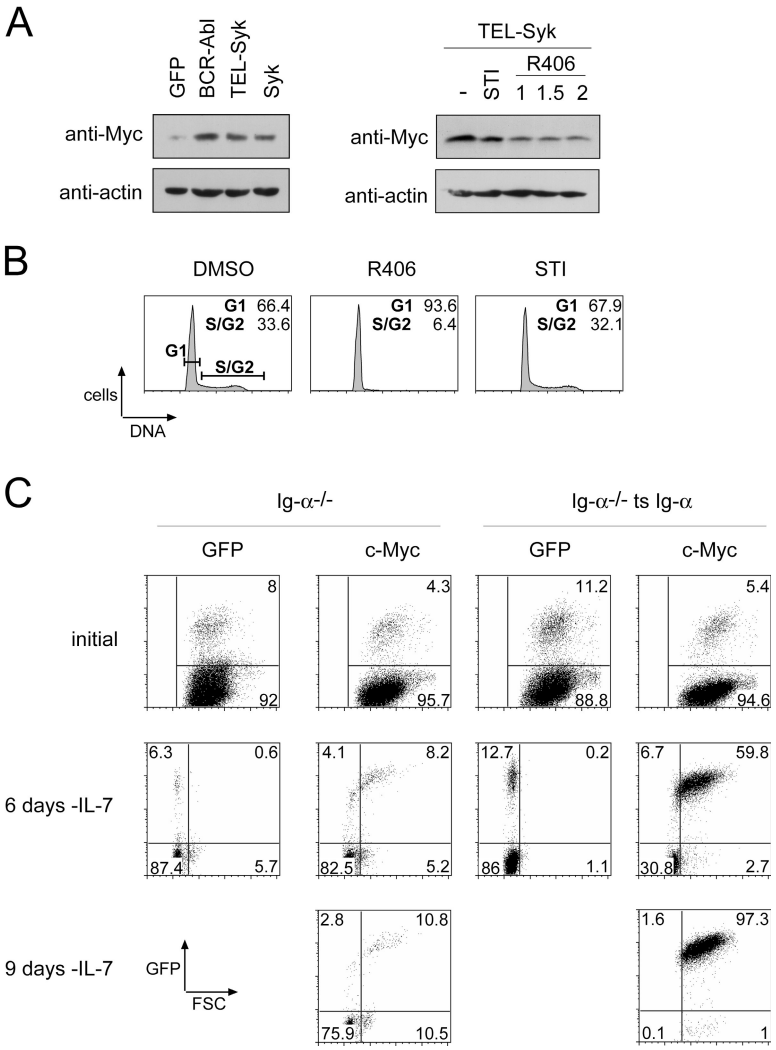

Figure 7. Transformation by Syk induces c-Myc up-regulation in pre–B cells. (A, left) Syk-transformed pre–B cells show augmented c-Myc expression. Immunoblot analysis of pre–B cells transduced with IRES-GFP vectors for the expression of GFP, Syk, TEL-Syk, or BCR-Abl. Data are representative of four independent experiments. (right) Inhibition of Syk decreases c-Myc expression in TEL-Syk–transformed cells. Immunoblot analysis of pre–B cells transformed by TEL-Syk treated with DMSO, 2 μM STI-571 for 2 d, or 2 μM R406 for 1, 1.5, and 2 d, as indicated. Data are representative of two independent experiments. (B) Proliferation of c-Myc–transformed pre–B cells depends on Syk activity. Histograms showing DNA content of c-Myc–transformed cells that were treated for 36 h with DMSO, 2 μM R406, or 2 μM STI-571. Data are representative of more than five independent experiments. (C) Pre-BCR expression is required for the transformation of Ig-$\alpha^{-/-}$ pro–B cells by c-Myc. FACS profiles show Ig-$\alpha^{-/-}$ pro–B cells (left) or Ig-$\alpha^{-/-}$ cells reconstituted with Ig-α (right). Cells were retrovirally transduced with IRES-GFP vectors expressing either GFP alone or c-Myc, cultured in the absence of IL-7, and analyzed at days 1, 6, and 9 after transduction. Numbers represent the percentage of cells in the indicated quadrant or gate. FSC, forward scatter.

in pre–B cells, we examined whether the presence of the pre-BCR is required for c-Myc–induced pre–B cell transformation. Therefore, we retrovirally expressed c-Myc in RAG-$2^{-/-}$ or Ig-$\alpha^{-/-}$ pro–B cells, both of which lack pre-BCR expression. Remarkably, neither RAG-$2^{-/-}$ nor Ig-$\alpha^{-/-}$ pro–B cells could be transformed by c-Myc overexpression (Fig. 7 C and not depicted). Even though Ig-$\alpha^{-/-}$ cells expressing c-Myc showed a slightly prolonged survival in the absence of IL-7, they failed to proliferate and did not give rise to stable cell lines (Fig. 7 C). However, reconstitution of pre-BCR expression by retroviral expression of Ig-α led to strong expansion of c-Myc–expressing cells that yielded stable IL-7–independent lines (Fig. 7 C). These data demonstrate that pre-BCR signaling is required for the proliferation of c-Myc–transformed cells. In line with this, reconstitution of pre-BCR expression in Ig-$\alpha^{-/-}$ cells increased the overall phosphotyrosine levels, whereas treatment with R406 reduced the overall phosphorylation in pre-BCR–expressing c-Myc–transformed cells (Fig. S2 C and not depicted).

Collectively, these data demonstrate that Syk-mediated pre-BCR signals are also required for the proliferation of

tumorigenic cells transformed by other oncogenes, increasing the potential of Syk as suitable target for the treatment of leukemia.

DISCUSSION

The data presented in this study suggest that the tyrosine kinase Syk is involved in pre–B cell transformation and leukemia development. By comparing Syk to the tumor suppressor SLP-65, we show that Syk does not reduce pre–B cell proliferation but rather transforms pre–B cell lines in a manner dependent on Syk kinase activity. Our results indicate that Syk-dependent transformation may be induced either by mutations directly affecting the Syk gene or by mutations that depend on Syk activity to transform particular cells. Although this implies that Syk is a protooncogene, some previous studies suggested a tumor suppressor role for Syk. For instance, Syk was not detected in invasive breast carcinoma tissues and seemed to be required for the suppression of tumor growth and metastasis in this cancer disease (27, 28). However, extended analysis of more breast cancer samples could not confirm these results, and an earlier report even demonstrated a correlation between Syk expression and breast cancer development, arguing against a tumor suppressor role for Syk (32, 43). In contrast, several lines of evidence indicate that Syk is a protooncogene. In agreement with this, recent studies show that Syk is expressed and constitutively phosphorylated in samples from ALL patients and that Syk gene amplification is found in mantle cell lymphomas (21–23). Furthermore, activation of Syk by ectopic expression of ITAM-bearing proteins was shown to transform fibroblasts, whereas counteracting Syk activity by pharmaceutical inhibition or expression of the regulatory phosphatase PTPROt was shown to inhibit proliferation of B lymphoma cells (24, 26, 44). Collectively, these data strongly suggest that deregulation of Syk is involved in malignant transformation and cancer development in humans and that expression and activation of Syk must be tightly controlled to allow proper signaling and prevent transformation. Consistent with this, we show that overexpression of Syk is not sufficient to transform freshly isolated BM-derived pre–B cells, most likely because these cells contain the appropriate mechanisms to regulate Syk activity. On the other hand, several immortalized pre–B cell lines could be efficiently transformed by Syk, presumably owing to preexisting mutations that may influence cell growth and facilitate transformation. Indeed, cell lines that could be easily transformed by Syk, although dependent on IL-7 as a growth factor, survived slightly longer in the absence of IL-7 than those lines that did not become growth factor–independent by overexpression of Syk (unpublished data). It is conceivable that mutations in genes regulating Syk activity provide a suitable background to allow Syk-mediated transformation. One example for a negative regulator of Syk is the adaptor protein Cbl. Upon phosphorylation by Syk, Cbl interacts with several molecules, including nonreceptor protein tyrosine kinases and adaptor proteins (45–48). However, Cbl also selectively binds to activated Syk via its RING finger domain, thereby targeting Syk to ubiquitinylation-mediated degradation (49, 50). Thus, Cbl-dependent degradation prevents sustained activation of signaling pathways involved in Syk-induced proliferation. Consequently, $Cbl^{-/-}$ mice develop splenomegaly, and the viral form v-Cbl lacking the RING finger domain is a potent oncogene (51–53).

The adaptor protein SLP-65 is, although more indirectly, also involved in the regulation of Syk. Upon activation, Syk phosphorylates SLP-65, thereby leading to the formation of a signaling complex that subsequently induces internalization of the pre-BCR. This mechanism prevents sustained activation of Syk by ITAM-bearing pre-BCR complexes expressed on the cell surface and, consequently, stops proliferation and enables differentiation. Hence, loss of SLP-65 leads to the expression of high amounts of pre- BCR on the cell surface, thereby providing constant proliferation signals mediated by Syk. In line with this, ~6% of $SLP\text{-}65^{-/-}$ mice develop leukemia, and loss of SLP-65 expression was shown in human leukemia disease (11, 12).

In addition to mutations that modulate Syk activity indirectly, other mutations may directly affect Syk expression or function. One example is the TEL-Syk fusion protein that was originally isolated from a patient with myelodysplastic syndrome (18). In TEL-Syk, the dimerization domain of the transcription factor TEL is fused to the linker region of Syk, and TEL-induced dimerization is thought to augment Syk autophosphorylation, thereby leading to constitutive activation (18, 19). A further translocation isolated from 5 out of 30 tested patients with heterogeneous peripheral T cell lymphoma fuses the N-terminal PH domain and the proline-rich Tec homology domain of ITK to the linker region of Syk (20). Although the breakpoints are not identical in TEL-Syk and ITK-Syk fusions, they are within the same region of the Syk gene, suggesting that this part is a hot spot for chromosomal translocations. The exact mechanism by which these fusion proteins induce cellular transformation and leukemia development remains to be elucidated. However, we found the lipid-modifying enzymes PLCγ2 and PI3-K to be constitutively phosphorylated upon Syk-induced transformation. Both molecules were shown to be direct substrates of Syk and are involved in the activation of survival- and proliferation-inducing pathways, suggesting that transformation by Syk, at least partially, depends on these enzymes (19, 54, 55). In particular, transformation by ITK-Syk might be explained by a PI3-K–dependent mechanism, where the PH domain of ITK mediates the recruitment of the ITK-Syk fusion protein to the plasma membrane by binding to phosphatidylinositol $(3,4,5)P_3$. This would bring the Syk kinase domain into the proximity of membrane-associated PI3-K, which, upon activation by Syk, catalyzes the production of more phosphatidyl inositol$(3,4,5)P_3$, thereby establishing a positive feedback activation of both Syk and PI3-K. Activation of the PI3-K pathway was previously shown to up-regulate expression of the transcription factor c-Myc in B lymphocytes (56). In agreement with the constitutive phosphorylation of PI3-K, we found elevated levels of c-Myc protein in Syk-transformed

cells, suggesting that up-regulation of c-Myc is a key event in Syk-induced transformation. However, our data also show that elevated expression of c-Myc is not sufficient for the transformation of pre–B cells and that additional pre-BCR–derived proliferation signals mediated via Syk are involved. Specifically, we show that reconstitution of pre-BCR expression increases the overall tyrosine phosphorylation in Ig-$\alpha^{-/-}$ cells and allows c-Myc–mediated transformation, whereas blocking pre-BCR signaling by inhibition of Syk or Src family kinases prevents proliferation. On the other hand, we show that transformation by TEL-Syk does not depend on pre-BCR expression and Src kinases, suggesting that constitutively active TEL-Syk, besides up-regulating c-Myc, mimics pre-BCR–derived proliferation signals.

Although our data show that Syk is required for proliferation and that deregulated Syk activity results in pre–B cell transformation, they do not rule out a role for Syk in differentiation. In fact, by phosphorylating SLP-65, Syk is involved in the activation of pre–B cell differentiation. Defective SLP-65 activation may explain the developmental block of Syk$^{-/-}$ pre–B cells (57–59) and their increased in vitro proliferation rate as compared with WT cells (60). In these Syk$^{-/-}$ pre–B cells, ZAP-70, a Syk family kinase, may replace Syk in inducing proliferation. However, additional experiments are required to determine the proliferation rate of Syk$^{-/-}$ and Syk$^{-/-}$/ZAP-70$^{-/-}$ pre–B cells compared with WT counterparts.

Together with previously published studies, our data suggest that deregulated Syk kinase activity may be a frequent mechanism for the transformation of lymphocytes and the development of leukemia. Moreover, we demonstrate that Syk kinase activity is required for the proliferation of pre–B cells transformed by other oncogenes such as c-Myc and that treatment of those tumorigenic cells with the novel Syk kinase inhibitor R406 blocks proliferation. Therefore, specific kinase inhibitors for Syk may provide a potential approach for the treatment of leukemia in human.

MATERIALS AND METHODS

Mice. RAG-2/γC$^{-/-}$ mice (61) were used for adoptive transfer experiments. BM cells from BALB/c mice were retrovirally transduced with the respective vectors, and 1–5 $\times$ 10^6 cells were injected into RAG-2/γC$^{-/-}$ mice 1 wk after transduction. When showing the first symptoms of disease, mice were killed for analysis of spleen and BM. RAG-2/γC$^{-/-}$ and BALB/c (WT) mice were bred at the animal facility of the Max-Planck-Institute for Immunobiology. Animal experiments were done in compliance with guidelines of the German law and the Max-Planck-Institute for Immunobiology.

Cell culture. Cell lines were grown in Iscove's medium (Biochrom) containing 10% FCS (Vitromex), 2 mM L-glutamine, 100 U/ml penicillin, 100 U/ml streptomycin (Invitrogen), and 50 μM 2-mercaptoethanol. For culture of IL-7–dependent cells, culture supernatant from J558L cells stably transfected with a mouse IL-7 expression vector was added. BM cells were isolated from 6–12-wk-old BALB/c mice and kept in culture for at least 1 d before being subjected to experiments.

Plasmids and retroviral transduction. Retroviral transductions were performed as described previously (62). The retroviral expression vector pMIG contains an IRES followed by the GFP cDNA. pMIG-BCR-Abl was a gift from W.S. Pear (University of Pennsylvania, Philadelphia, PA). pMIG-Syk, pMIG-TEL-Syk, and pMIG-Myc were generated by insertion of PCR fragments containing the complete open reading frame of human Syk, TEL-Syk, or c-Myc into pMIG. Mutant TEL-SykK402A was generated by PCR using primers carrying the desired mutation and primers covering the 5′ and 3′ ends of TEL and Syk, respectively. Vectors encoding TEL-Syk and BCR-Abl without fluorescence markers were generated by excision of the IRES-GFP sequence and religation of the respective vectors. For construction of the recombination reporter plasmid, we ligated a PCR fragment including both 5′ and 3′ RSS amplified from the vector pJH288 (63) into the retroviral expression vector pMOWS containing a puromycin resistance gene for selection (62). GFP cDNA was ligated in between the RSS in reverse orientation.

Flow cytometry and cell sorting. Cells were stained for FACS with anti-CD19 (BD Biosciences), anti-κ (Southern Biotechnology Associates, Inc.), anti-IgM (Jackson ImmunoResearch Laboratories), and anti-SLC/HC (SL156; a gift from Ton Rolink, University of Basel, Basel, Switzerland). For cell cycle analysis, cells were fixed in 70% ethanol overnight and subsequently incubated in PBS containing 1.6 μg/ml propidium iodide and 1 μg/ml RNase for 30 min at 37°C. Acquisition was performed with the LSR II (Becton Dickinson). Transduced GFP$^+$ cells were sorted to a purity >99% using a high-speed sorter (MoFlo; Cytomation).

Cell lysis and immunoblotting. TEL-Syk– and BCR-Abl–expressing cells were grown in medium without IL-7. GFP- and TEL-SykK402A–expressing cells were sorted for GFP expression, expanded in medium containing IL-7, and cultured for 24 h in the absence of IL-7. Cells were lysed in 5 μl lysis buffer (50 mM Tris-HCl, pH 7.4, 1% Triton [Sigma-Aldrich], 137.5 mM NaCl, 1% glycerol, 1 mM Na-orthovanadate, 0.5 mM EDTA, pH 8, and protease inhibitor cocktail [Sigma-Aldrich]) per 10^5 cells. Lysates were separated on 10% SDS-polyacrylamide gels and transferred onto nitrocellulose membranes (Hybond; GE Healthcare). Membranes were blocked with 5% milk powder in PBT (PBS, 0.1% Tween 20) for 1 h. Primary antibodies were diluted in PBT supplemented with 2% bovine serum albumin fraction V (BIOMOL Research Laboratories, Inc.), and secondary antibodies were diluted in blocking solution. Immunoreactive proteins were detected using a chemolumiscence detection system (ECL; GE Healthcare). Antibodies used were anti-Syk (4D10; Santa Cruz Biotechnology, Inc.), anti-Syk (N19; Santa Cruz Biotechnology, Inc.), anti–SLP-65 (generated in our lab), antiphosphotyrosine (4G10; Upstate Biotechnology), anti–α-actin (I-19; Santa Cruz Biotechnology, Inc.), anti–Ig-α (64), anti-cAbl (K-12; Santa Cruz Biotechnology, Inc.), and anti–c-Myc (9E10).

Application of kinase inhibitors. Syk inhibitor R406 (provided by Rigel Pharmaceuticals, Inc., San Francisco, CA), Abl inhibitor STI-571 (provided by Novartis, Basel, Switzerland), Src inhibitor PP2 (Calbiochem), and Syk inhibitor piceatannol (Sigma-Aldrich) were dissolved in DMSO. For treatment, cells were cultured in medium containing 0.5% DMSO and either 2 μM R406, 2 μM STI-571, 5 μM PP2, or 50 μM piceatannol.

Mass spectrometry. Immobilized antiphosphotyrosine antibody (4G10) was used to precipitate 10^6 nontransformed, Tel-Syk, and BCR-Abl–transformed cells, respectively. Bound proteins were resolved by 10% SDS-PAGE and visualized by silver staining. Individual bands were excised and enzymatically digested with trypsin. Before LC-MS analysis, tryptic peptide mixtures were desalted using STAGE tips as described previously (65). Nanoscale LC (MDLC; GE Healthcare) was coupled to a 7-tesla linear ion-trap Fourier-transform ion cyclotron resonance mass spectrometer (LTQ-FT; Thermo Electron) equipped with a nanoelectrospray source (Proxeon). Peptides were eluted from an analytical column by a linear gradient running from 2 to 60% (vol/vol) acetonitrile (in 0.5% acetic acid) with a flow rate of 250 nl/min in 35 min and sprayed directly into the aperture of the mass spectrometer. Information-dependent acquisition of MS, MS/MS, and MS^3 spectra was performed as described previously (66). Acquired spectra were

then searched with Mascot (Matrix Science) against the human and mouse International Protein Index protein database (version 3.14, available at http://www.ebi.ac.uk/IPI/), to which we added frequently observed contaminants. Tryptic enzyme specificity with up to two missed cleavages was applied to all searches. Protein identifications were further analyzed and manually verified by the use of MSQuant (available at http://msquant.sourceforge.net).

Online supplemental material. Fig. S1 shows the reduction of Syk-induced overall tyrosine phosphorylation after treatment with R406, whereas tyrosine phosphorylation in BCR-Abl–transformed cells is unaffected by R406 treatment. Fig. S2 shows that overexpression of c-Myc leads to the transformation of pre–B cells and that pre-BCR signaling is required for c-Myc–induced transformation. Online supplemental material is available at http://www.jem.org/cgi/content/full/jem.20060967/DC1.

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REFERENCES

1. Hardy, R.R., C.E. Carmack, S.A. Shinton, J.D. Kemp, and K. Hayakawa. 1991. Resolution and characterization of pro-B and pre–pro-B cell stages in normal mouse bone marrow. *J. Exp. Med.* 173:1213–1225.
2. Rolink, A.G., E. ten Boekel, T. Yamagami, R. Ceredig, J. Andersson, and F. Melchers. 1999. B cell development in the mouse from early progenitors to mature B cells. *Immunol. Lett.* 68:89–93.
3. Karasuyama, H., A. Rolink, Y. Shinkai, F. Young, F.W. Alt, and F. Melchers. 1994. The expression of Vpre-B/lambda 5 surrogate light chain in early bone marrow precursor B cells of normal and B cell-deficient mutant mice. *Cell.* 77:133–143.
4. Tsubata, T., and M. Reth. 1990. The products of pre–B cell–specific genes (λ_5 and V_{preB}) and the immunoglobulin μ chain form a complex that is transported onto the cell surface. *J. Exp. Med.* 172:973–976.
5. Rolink, A.G., T. Winkler, F. Melchers, and J. Andersson. 2000. Precursor B cell receptor–dependent B cell proliferation and differentiation does not require the bone marrow or fetal liver environment. *J. Exp. Med.* 191:23–32.
6. Burrows, P.D., R.P. Stephan, Y.H. Wang, K. Lassoued, Z. Zhang, and M.D. Cooper. 2002. The transient expression of pre-B cell receptors governs B cell development. *Semin. Immunol.* 14:343–349.
7. Ohnishi, K., and F. Melchers. 2003. The nonimmunoglobulin portion of lambda5 mediates cell-autonomous pre-B cell receptor signaling. *Nat. Immunol.* 4:849–856.
8. Niiro, H., and E.A. Clark. 2002. Regulation of B-cell fate by antigen-receptor signals. *Nat. Rev. Immunol.* 2:945–956.
9. Sada, K., T. Takano, S. Yanagi, and H. Yamamura. 2001. Structure and function of Syk protein-tyrosine kinase. *J. Biochem. (Tokyo).* 130:177–186.
10. Koretzky, G.A., F. Abtahian, and M.A. Silverman. 2006. SLP76 and SLP65: complex regulation of signalling in lymphocytes and beyond. *Nat. Rev. Immunol.* 6:67–78.
11. Jumaa, H., L. Bossaller, K. Portugal, B. Storch, M. Lotz, A. Flemming, M. Schrappe, V. Postila, P. Riikonen, J. Pelkonen, et al. 2003. Deficiency of the adaptor SLP-65 in pre-B-cell acute lymphoblastic leukaemia. *Nature.* 423:452–456.
12. Flemming, A., T. Brummer, M. Reth, and H. Jumaa. 2003. The adaptor protein SLP-65 acts as a tumor suppressor that limits pre-B cell expansion. *Nat. Immunol.* 4:38–43.
13. Hayashi, K., M. Yamamoto, T. Nojima, R. Goitsuka, and D. Kitamura. 2003. Distinct signaling requirements for Dmu selection, IgH allelic exclusion, pre-B cell transition, and tumor suppression in B cell progenitors. *Immunity.* 18:825–836.
14. Kurzrock, R., H.M. Kantarjian, B.J. Druker, and M. Talpaz. 2003. Philadelphia chromosome-positive leukemias: from basic mechanisms to molecular therapeutics. *Ann. Intern. Med.* 138:819–830.
15. Popescu, N.C., and D.B. Zimonjic. 2002. Chromosome-mediated alterations of the MYC gene in human cancer. *J. Cell. Mol. Med.* 6:151–159.
16. Adhikary, S., and M. Eilers. 2005. Transcriptional regulation and transformation by Myc proteins. *Nat. Rev. Mol. Cell Biol.* 6:635–645.
17. Dalla-Favera, R., M. Bregni, J. Erikson, D. Patterson, R.C. Gallo, and C.M. Croce. 1982. Human c-myc onc gene is located on the region of chromosome 8 that is translocated in Burkitt lymphoma cells. *Proc. Natl. Acad. Sci. USA.* 79:7824–7827.
18. Kuno, Y., A. Abe, N. Emi, M. Iida, T. Yokozawa, M. Towatari, M. Tanimoto, and H. Saito. 2001. Constitutive kinase activation of the TEL-Syk fusion gene in myelodysplastic syndrome with t(9;12)(q22;p12). *Blood.* 97:1050–1055.
19. Kanie, T., A. Abe, T. Matsuda, Y. Kuno, M. Towatari, T. Yamamoto, H. Saito, N. Emi, and T. Naoe. 2004. TEL-Syk fusion constitutively activates PI3-K/Akt, MAPK and JAK2-independent STAT5 signal pathways. *Leukemia.* 18:548–555.
20. Streubel, B., U. Vinatzer, M. Willheim, M. Raderer, and A. Chott. 2006. Novel t(5;9)(q33;q22) fuses ITK to SYK in unspecified peripheral T-cell lymphoma. *Leukemia.* 20:313–318.
21. Guillaume, N., C. Alleaume, D. Munfus, J.C. Capiod, G. Touati, B. Pautard, B. Desablens, J.J. Lefrere, F. Gouilleux, K. Lassoued, and V. Gouilleux-Gruart. 2005. ZAP-70 tyrosine kinase is constitutively expressed and phosphorylated in B-lineage acute lymphoblastic leukemia cells. *Haematologica.* 90:899–905.
22. Chiaretti, S., A. Guarini, M.S. De Propris, S. Tavolaro, S. Intoppa, A. Vitale, S. Iacobelli, L. Elia, C. Ariola, J. Ritz, and R. Foa. 2006. ZAP-70 expression in acute lymphoblastic leukemia: association with the E2A/PBX1 rearrangement and the pre-B stage of differentiation and prognostic implications. *Blood.* 107:197–204.
23. Rinaldi, A., I. Kwee, M. Taborelli, C. Largo, S. Uccella, V. Martin, G. Poretti, G. Gaidano, G. Calabrese, G. Martinelli, et al. 2006. Genomic and expression profiling identifies the B-cell associated tyrosine kinase Syk as a possible therapeutic target in mantle cell lymphoma. *Br. J. Haematol.* 132:303–316.
24. Grande, S.M., E. Katz, J.E. Crowley, M.S. Bernardini, S.R. Ross, and J.G. Monroe. 2006. Cellular ITAM-containing proteins are oncoproteins in nonhematopoietic cells. *Oncogene.* 25:2748–2757.
25. Katz, E., M.H. Lareef, J.C. Rassa, S.M. Grande, L.B. King, J. Russo, S.R. Ross, and J.G. Monroe. 2005. MMTV Env encodes an ITAM responsible for transformation of mammary epithelial cells in three-dimensional culture. *J. Exp. Med.* 201:431–439.
26. Chen, L., P. Juszczynski, K. Takeyama, R.C.T. Aguiar, and M.A. Shipp. 2006. Protein tyrosine phosphatase receptor–type O truncated (PTPROt) regulates SYK phosphorylation, proximal B-Cell–receptor signaling, and cellular proliferation. *Blood.* 108:3428–3433.
27. Coopman, P.J., M.T. Do, M. Barth, E.T. Bowden, A.J. Hayes, E. Basyuk, J.K. Blancato, P.R. Vezza, S.W. McLeskey, P.H. Mangeat, and S.C. Mueller. 2000. The Syk tyrosine kinase suppresses malignant growth of human breast cancer cells. *Nature.* 406:742–747.
28. Moroni, M., V. Soldatenkov, L. Zhang, Y. Zhang, G. Stoica, E. Gehan, B. Rashidi, B. Singh, M. Ozdemirli, and S.C. Mueller. 2004. Progressive loss of Syk and abnormal proliferation in breast cancer cells. *Cancer Res.* 64:7346–7354.
29. Toyama, T., H. Iwase, H. Yamashita, Y. Hara, Y. Omoto, H. Sugiura, Z. Zhang, and Y. Fujii. 2003. Reduced expression of the Syk gene is correlated with poor prognosis in human breast cancer. *Cancer Lett.* 189:97–102.
30. Wang, S., Y.B. Ding, G.Y. Chen, J.G. Xia, and Z.Y. Wu. 2004. Hypermethylation of Syk gene in promoter region associated with oncogenesis and metastasis of gastric carcinoma. *World J. Gastroenterol.* 10:1815–1818.

31. Hoeller, C., C. Thallinger, B. Pratscher, M.D. Bister, N. Schicher, R. Loewe, E. Heere-Ress, F. Roka, V. Sexl, and H. Pehamberger. 2005. The non-receptor-associated tyrosine kinase Syk is a regulator of metastatic behavior in human melanoma cells. *J. Invest. Dermatol.* 124:1293–1299.
32. Elkak, A., W. Al Sarakbi, and K. Mokbel. 2005. SYK expression in human breast cancer. *J. Carcinog.* 4:7.
33. Goodman, P.A., C.M. Wood, A. Vassilev, C. Mao, and F.M. Uckun. 2001. Spleen tyrosine kinase (Syk) deficiency in childhood pro-B cell acute lymphoblastic leukemia. *Oncogene.* 20:3969–3978.
34. Takata, M., H. Sabe, A. Hata, T. Inazu, Y. Homma, T. Nukada, H. Yamamura, and T. Kurosaki. 1994. Tyrosine kinases Lyn and Syk regulate B cell receptor-coupled Ca2+ mobilization through distinct pathways. *EMBO J.* 13:1341–1349.
35. Cha, H.S., D.L. Boyle, T. Inoue, R. Schoot, P.P. Tak, P. Pine, and G.S. Firestein. 2006. A novel spleen tyrosine kinase inhibitor blocks c-Jun N-terminal kinase-mediated gene expression in synoviocytes. *J. Pharmacol. Exp. Ther.* 317:571–578.
36. Matsubara, S., G. Li, K. Takeda, J.E. Loader, P. Pine, E.S. Masuda, N. Miyahara, S. Miyahara, J.J. Lucas, A. Dakhama, and E.W. Gelfand. 2006. Inhibition of spleen tyrosine kinase prevents mast cell activation and airway hyperresponsiveness. *Am. J. Respir. Crit. Care Med.* 173:56–63.
37. Braselmann, S., V. Taylor, S. Wang, C. Sylvain, M. Balloum, K. Qu, E. Herlaar, A. Lau, C. Young, H. Zhao, et al. 2006. R406, an Orally Available Syk Kinase Inhibitor Blocks Fc Receptor Signaling and Reduces Immune Complex-Mediated Inflammation. *J. Pharmacol. Exp. Ther.* 10.1124/jpet.106.109058.
38. Sawyers, C.L., W. Callahan, and O.N. Witte. 1992. Dominant negative MYC blocks transformation by ABL oncogenes. *Cell.* 70:901–910.
39. Wong, K.K., J.D. Hardin, S. Boast, C.L. Cooper, K.T. Merrell, T.G. Doyle, S.P. Goff, and K.L. Calame. 1995. A role for c-Abl in c-myc regulation. *Oncogene.* 10:705–711.
40. Kurosaki, T., M. Takata, Y. Yamanashi, T. Inazu, T. Taniguchi, T. Yamamoto, and H. Yamamura. 1994. Syk activation by the Src-family tyrosine kinase in the B cell receptor signaling. *J. Exp. Med.* 179:1725–1729.
41. Keshvara, L.M., C.C. Isaacson, T.M. Yankee, R. Sarac, M.L. Harrison, and R.L. Geahlen. 1998. Syk- and Lyn-dependent phosphorylation of Syk on multiple tyrosines following B cell activation includes a site that negatively regulates signaling. *J. Immunol.* 161:5276–5283.
42. El-Hillal, O., T. Kurosaki, H. Yamamura, J.P. Kinet, and A.M. Scharenberg. 1997. syk kinase activation by a src kinase-initiated activation loop phosphorylation chain reaction. *Proc. Natl. Acad. Sci. USA.* 94:1919–1924.
43. Fluck, M., G. Zurcher, A.C. Andres, and A. Ziemiecki. 1995. Molecular characterization of the murine syk protein tyrosine kinase cDNA, transcripts and protein. *Biochem. Biophys. Res. Commun.* 213:273–281.
44. Leseux, L., S.M. Hamdi, T. Al Saati, F. Capilla, C. Recher, G. Laurent, and C. Bezombes. 2006. Syk-dependent mTOR activation in follicular lymphoma cells. *Blood.* 10.1182/blood-2006-05-026203.
45. Panchamoorthy, G., T. Fukazawa, S. Miyake, S. Soltoff, K. Reedquist, B. Druker, S. Shoelson, L. Cantley, and H. Band. 1996. p120cbl is a major substrate of tyrosine phosphorylation upon B cell antigen receptor stimulation and interacts in vivo with Fyn and Syk tyrosine kinases, Grb2 and Shc adaptors, and the p85 subunit of phosphatidylinositol 3-kinase. *J. Biol. Chem.* 271:3187–3194.
46. Meisner, H., B.R. Conway, D. Hartley, and M.P. Czech. 1995. Interactions of Cbl with Grb2 and phosphatidylinositol 3′-kinase in activated Jurkat cells. *Mol. Cell. Biol.* 15:3571–3578.
47. Fukazawa, T., K.A. Reedquist, T. Trub, S. Soltoff, G. Panchamoorthy, B. Druker, L. Cantley, S.E. Shoelson, and H. Band. 1995. The SH3 domain-binding T cell tyrosyl phosphoprotein p120. Demonstration of its identity with the c-cbl protooncogene product and in vivo complexes with Fyn, Grb2, and phosphatidylinositol 3-kinase. *J. Biol. Chem.* 270:19141–19150.
48. Cory, G.O., R.C. Lovering, S. Hinshelwood, L. MacCarthy-Morrogh, R.J. Levinsky, and C. Kinnon. 1995. The protein product of the c-cbl protooncogene is phosphorylated after B cell receptor stimulation and binds the SH3 domain of Bruton's tyrosine kinase. *J. Exp. Med.* 182:611–615.
49. Rao, N., A.K. Ghosh, S. Ota, P. Zhou, A.L. Reddi, K. Hakezi, B.K. Druker, J. Wu, and H. Band. 2001. The non-receptor tyrosine kinase Syk is a target of Cbl-mediated ubiquitylation upon B-cell receptor stimulation. *EMBO J.* 20:7085–7095.
50. Sohn, H.W., H. Gu, and S.K. Pierce. 2003. Cbl-b negatively regulates B cell antigen receptor signaling in mature B cells through ubiquitination of the tyrosine kinase Syk. *J. Exp. Med.* 197:1511–1524.
51. Murphy, M.A., R.G. Schnall, D.J. Venter, L. Barnett, I. Bertoncello, C.B. Thien, W.Y. Langdon, and D.D. Bowtell. 1998. Tissue hyperplasia and enhanced T-cell signalling via ZAP-70 in c-Cbl-deficient mice. *Mol. Cell. Biol.* 18:4872–4882.
52. Langdon, W.Y., J.W. Hartley, S.P. Klinken, S.K. Ruscetti, and H.C. Morse III. 1989. v-cbl, an oncogene from a dual-recombinant murine retrovirus that induces early B-lineage lymphomas. *Proc. Natl. Acad. Sci. USA.* 86:1168–1172.
53. Blake, T.J., M. Shapiro, H.C. Morse III, and W.Y. Langdon. 1991. The sequences of the human and mouse c-cbl proto-oncogenes show v-cbl was generated by a large truncation encompassing a proline-rich domain and a leucine zipper-like motif. *Oncogene.* 6:653–657.
54. Marshall, A.J., H. Niiro, T.J. Yun, and E.A. Clark. 2000. Regulation of B-cell activation and differentiation by the phosphatidylinositol 3-kinase and phospholipase Cgamma pathway. *Immunol. Rev.* 176:30–46.
55. Beitz, L.O., D.A. Fruman, T. Kurosaki, L.C. Cantley, and A.M. Scharenberg. 1999. SYK is upstream of phosphoinositide 3-kinase in B cell receptor signaling. *J. Biol. Chem.* 274:32662–32666.
56. Grumont, R.J., A. Strasser, and S. Gerondakis. 2002. B cell growth is controlled by phosphatidylinosotol 3-kinase-dependent induction of Rel/NF-kappaB regulated c-myc transcription. *Mol. Cell.* 10:1283–1294.
57. Cheng, A.M., B. Rowley, W. Pao, A. Hayday, J.B. Bolen, and T. Pawson. 1995. Syk tyrosine kinase required for mouse viability and B-cell development. *Nature.* 378:303–306.
58. Turner, M., P.J. Mee, P.S. Costello, O. Williams, A.A. Price, L.P. Duddy, M.T. Furlong, R.L. Geahlen, and V.L. Tybulewicz. 1995. Perinatal lethality and blocked B-cell development in mice lacking the tyrosine kinase Syk. *Nature.* 378:298–302.
59. Saijo, K., C. Schmedt, I.H. Su, H. Karasuyama, C.A. Lowell, M. Reth, T. Adachi, A. Patke, A. Santana, and A. Tarakhovsky. 2003. Essential role of Src-family protein tyrosine kinases in NF-kappaB activation during B cell development. *Nat. Immunol.* 4:274–279.
60. Siegel, R., U. Kim, A. Patke, X. Yu, X. Ren, A. Tarakhovsky, and R.G. Roeder. 2006. Nontranscriptional regulation of SYK by the coactivator OCA-B is required at multiple stages of B cell development. *Cell.* 125:761–774.
61. Colucci, F., C. Soudais, E. Rosmaraki, L. Vanes, V.L. Tybulewicz, and J.P. Di Santo. 1999. Dissecting NK cell development using a novel alymphoid mouse model: investigating the role of the c-abl proto-oncogene in murine NK cell differentiation. *J. Immunol.* 162:2761–2765.
62. Ketteler, R., S. Glaser, O. Sandra, U.M. Martens, and U. Klingmuller. 2002. Enhanced transgene expression in primitive hematopoietic progenitor cells and embryonic stem cells efficiently transduced by optimized retroviral hybrid vectors. *Gene Ther.* 9:477–487.
63. Hesse, J.E., M.R. Lieber, M. Gellert, and K. Mizuuchi. 1987. Extrachromosomal DNA substrates in pre-B cells undergo inversion or deletion at immunoglobulin V-(D)-J joining signals. *Cell.* 49:775–783.
64. Mielenz, D., A. Ruschel, C. Vettermann, and H.M. Jack. 2003. Immunoglobulin mu heavy chains do not mediate tyrosine phosphorylation of Ig alpha from the ER-cis-Golgi. *J. Immunol.* 171:3091–3101.
65. Rappsilber, J., Y. Ishihama, and M. Mann. 2003. Stop and go extraction tips for matrix-assisted laser desorption/ionization, nanoelectrospray, and LC/MS sample pretreatment in proteomics. *Anal. Chem.* 75:663–670.
66. Olsen, J.V., and M. Mann. 2004. Improved peptide identification in proteomics by two consecutive stages of mass spectrometric fragmentation. *Proc. Natl. Acad. Sci. USA.* 101:13417–13422.